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NOAA-TM-NMFS-SWFSC-400

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
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Table of Contents

List of Tables

List of Figures

ABSTRACT

Procedures to obtain DNA from formalin-preserved samples exist; however, most have been based on paraffin-embedded tissues prepared for histological examination. Few studies have demonstrated the ability to obtain DNA directly from formalin-preserved tissues located in museums and historical collections. We tested three extraction protocols on formalin-preserved skin and teeth from the SWFSC's Marine Mammal Life History Collection. We were able to obtain small amounts of fragmented DNA using two protocols. Of 25 skin samples, only eight samples produced fragmented sequences, ranging from 70-210 bases of the mitochondrial (mt) control region. All nine teeth yielded sufficient DNA to amplify 200-400 bases of the mt control region, however, useable sequence could only be obtained from seven of the nine teeth. For several samples, there were also corresponding non-formalin preserved tissues available from which we obtained a 400 base sequence of the same mt control region. In comparing the two sequences generated from tissue from the same animal from the two different preservatives, several base mutations were discovered in the sequences generated from the formalin-preserved tissue. All were C/T or G/A mutations, which are consistent with previously observed mutations caused by formalin damage in sequences generated for human genetic research. This research demonstrates that care needs to be taken in interpreting sequence data generated from formalin-preserved tissues.

INTRODUCTION

The Southwest Fisheries Science Center (SWFSC) Marine Mammal Life History Collection is comprised mostly of small cetacean tissues collected from animals incidentally taken in the Eastern Tropical Pacific (ETP) tuna fishery and California drift/gillnet fishery (CAGN). The formalin-fixed samples collected from the ETP date back over 30 years and represent a potentially valuable resource for historical stock structure and biodiversity studies using molecular genetic techniques.

Formalin is used as a preservative due to its ability to permanently preserve a wide range of tissues, however, during the tissue fixation process, DNA is damaged. It is thought that formalin causes cross-linking between proteins and nucleic acids, creating difficulty in 'freeing up' the DNA during extraction procedures (Srinivasin et al. 2002). Formalin may also cause fragmentation of DNA and sequence modification (Tang 2006). Several studies have demonstrated the ability to obtain DNA from either fresh, formalin-preserved clinical samples or formalin preserved paraffin-embedded histological samples (Heller et al. 1991; Koshiba et al. 1993; Williams et al. 1999; Sato et al. 2001; Legrand et al. 2002; Shi et al. 2002; Quach et al. 2004). Few studies, however, have demonstrated the ability to obtain DNA directly from formalin-preserved tissues located in museums and historical collections (Shedlock et al. 1997; Schander and Halanych 2003).

Many museum collections hold a large number of formalin-preserved samples. Often these include voucher specimens or large historical collections that could provide valuable genetic information. The SWFSC holds one of the largest marine mammal life history collections in the world. The majority of the samples [jaw sections (removed from the head), gonads, adrenal glands, and stomach contents] were collected by biologists aboard fishing vessels in the Eastern Tropical Pacific tuna fishery or the California drift/gillnet fishery. The collection consists of over 30,000 samples dating back to the late 1960's. Samples were previously preserved in 10% buffered formalin solution for varying times and then transferred to a 70% isopropyl alcohol solution for permanent storage. Too see if this resource could be tapped for use in historical

stock structure studies, a small pilot project was undertaken to determine if usable DNA and mitochondrial control region sequence could be obtained from formalin-preserved tissues held at SWFSC.

METHODS

Formalin-preserved tissues were available from several different cetacean species. For this project 25 skin samples were selected including: four fresh skin biopsy samples collected and preserved immediately in formalin, two skin samples from stranded animals also preserved in formalin (previous samples not transferred to isopropyl alcohol), and 19 skin samples and nine teeth from the above mentioned life history collection. Sample details are summarized in Table 1.

Approximately 50-100mg of skin tissue was used for each extraction procedure. For samples used from the life history collection, skin was removed from jaw sections previously preserved in 10% buffered formalin for an unknown period of time and currently stored in 70% isopropyl alcohol. One to two teeth were also removed from select jaws. Prior to extraction, skin and teeth were placed in 1.5ml epitubes and soaked in 1ml of Milli-Q water with multiple rinse/soaks over the course of 24 hrs in order to remove any residual formalin or alcohol and to re-hydrate the tissue.

Three extraction methods were attempted. The first was an antigen retrieval (AR) method (Shi et al. 2002, modified at SWFSC by J. Hyde, Fisheries Resource Division) followed by a silica-based extraction protocol using Qiagen's QiaAmp kit (Qiagen Inc., Valencia, CA). The AR buffer is an alkaline solution that is thought to hydrolyze proteins and potentially break crosslinks between proteins and DNA caused by formalin fixation. The second extraction method, also silica based, was Höss and Pääbo (1993) developed for ancient DNA/bone work. The third method was a kit manufactured by Ambion Inc. (Ambion Inc., Austin, TX) called RecoverALL™ Total Nucleic Acid Isolation. The kit is optimized for use on formalin-fixed, paraffin-embedded tissues.

For skin, 24 samples were extracted using the AR method, three using the modified Höss and Pääbo method, and four using the RecoverALL kit. Sets of extractions were accompanied by two extraction controls to test for contamination in extraction reagents. Because yield of DNA

was expected to be low, DNA was not quantified by absorbance spectrophotometry. PCR was attempted on all DNA using 2-10ul of product. Several polymerases and PCR conditions were tried (see Table 2 for summary).

For teeth, nine samples were extracted using the Höss and Pääbo (1993) method. Extractions were performed in a 'clean room' specifically set up for bone/ancient DNA work. PCR was also performed in the 'clean room' using Biolase Taq (Bioline USA Inc., Boston, MA) and Restorase Taq (Sigma-Aldrich, St. Louis, MO) with the parameters listed in Table 2. One tooth extraction was attempted using the RecoverALL kit. PCR was performed using Biolase Taq.

DNA was amplified using several primer pairs to obtain sequence of varying length (Table 3). Standard protocols were used for PCR product cleaning (Qiagen Qiaquick PCR Purification Kit) and sequencing. Sequencing was done using the same primers as for amplification and Applied Biosystems Big Dye Terminator v3.1 (Applied Biosystems Inc., Foster City, CA). Sequencing products were run on an Applied Biosystems 3100 Genetic analyzer and sequences were aligned using the program Sequencher (v4.1, Gene Codes Corp., Ann Arbor, MI).

Extraction and PCR were replicated, following the procedures above, on separate days. Resulting sequences were checked for species accuracy by using GenBank® (NIH sequence database) or the SWFSC's cetacean reference sequence library.

For several of the formalin tooth samples, skin samples collected and preserved in a 20% salt saturated solution of DMSO (dimethyl sulfoxide) were also available from the same animal. As a further check, the accuracy of the sequences generated from the formalin tooth samples was compared with sequence generated from the DMSO preserved skin.

RESULTS AND CONCLUSIONS

Of the DNA extracted from the 25 skin samples, 12 amplified products of 250 bases or less, but only eight generated usable sequences. The antigen retrieval (AR) extraction method was found to be the most successful on skin, followed by PCR with Platinum® Taq (Invitrogen Corp., Carlsbad, CA). Neither the RecoverALL Kit (Platinum® Taq) nor the Höss and Pääbo

(1993) extraction (Biolase Taq) yielded DNA that generated good quality sequence from the skin samples.

DNA extracted from the nine teeth using the Höss and Pääbo (1993) method amplified for all samples, however, only seven of the nine yielded sequence of 250 to 400 bases. The single tooth extraction attempted with the RecoverALL Kit gave weak amplification of 150 bases and did not yield clean sequence. The higher success of obtaining DNA from the tooth material could be because DNA in hard material may be better protected from formalin damage (Tang 2006). Results of sequencing are shown in Table 4 along with the corresponding extraction method. All aligned sequences were confirmed to be the correct species.

Although 'successful' or 'usable' sequences were generated, some issues or problems were found. Several instances of ambiguous bases or bases that could not be called occurred in most sequences (Figure 1). The same ambiguities also occurred in replicate extraction and sequence runs. In addition, the results of the comparative sequences generated from the formalin teeth and DMSO-preserved skin did not agree. There is one case of an insertion/deletion shown in Figure 2 and, in Figure 3, base mutations are evident in the formalin sequences when compared to the sequences generated from the DMSO-preserved skin. The mutations in the formalin sequences are either C/T or G/A mutations and were consistent in replication.

Several previous studies on obtaining sequence from formalin-preserved clinical samples have demonstrated similar C/T or G/A mutations (Williams et al. 1999, Srinivasin et al. 2002, Quach et al. 2004). Researchers found that the DNA from formalin-preserved samples is often 'nicked' or damaged. A damaged base may halt PCR altogether or in some cases could cause the polymerase to travel across the damaged site, potentially putting an incorrect base in the damaged area, leaving an artificial mutation. Williams et al., 1999, speculates that the base mutations are caused by the formalin cross-linking cytosine nucleotides on either strand. During PCR, this causes the Taq polymerase to fail to recognize the cytosine, thus incorporating an adenine in the place of a guanosine, creating an artificial C/T or G/A mutation.

Previous studies have reported mutation rates of one base per 500-1000 bases (Williams et al, 1999, Srinivasin et al., 2002, Quach et al., 2004). The mutation rate for our results is much higher and randomly distributed (Table 5). The mutations occur more frequently at particular sites; however, they do not appear in all sequences. For one of the 200 base fragments, there were as many as eight mutations (4% mutation rate) and there were as many as 11 mutations in a 400 base fragment (2.75% mutation rate). It is possible that the higher mutation rate in these sequences is due to the tissue being stored longer in formalin than most clinical tissue samples. Tissues for histology are routinely preserved in formalin for 24-48 hours, with longer 'fixing' times adversely affecting the quality of DNA (Foss et al., 1994). The skin and teeth used in this study were in formalin for much longer time periods, ranging from one week to several months. Douglas and Rogers (1998) found that the average fragment size of DNA extracted from formalin-preserved tissue decreases with increasing fixation times, with DNA degradation occurring within the first three hours.

Although this study only examined a small number of samples, it has demonstrated that low molecular weight DNA can be obtained from formalin-fixed museum specimens. However, the mitochondrial control region sequences contained mutations caused by formalin damage, thus stressing the importance of confirming results with sequence from fresh-preserved tissues from the same specimen. If mutation and error rates can be determined, the sequences may still be usable in a given study provided the formalin-induced mutations do not introduce undue bias or noise to the data. A large number of sequences would need to be generated to determine true mutations and sequencing errors. It has been suggested that a minimum of four-times coverage would be necessary to ascertain the consistency of these errors (Tang 2006) so that they could be used reliably in a study. Tang 2006, which summarizes the proceeding of a workshop held specifically to discuss recovering DNA from formalin-fixed samples in museums, concluded that much work still needs to be done in order to ascertain the usefulness of these samples. Controlled experiments were suggested to try to determine the exact chemical effect of formalin on tissue and if it can be repaired. Other suggestions included a large number of replications and

cloning. In conclusion, further work and cooperative efforts need to be employed to determine accurate use of these valuable preserved museum collections.

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Table1. Species, tissue type, preservative and time in preservative for formalin-fixed samples. ETP Fishery is the Eastern tropical Pacific tuna fishery and CAGN Fishery is the California Drift/Gillnet Fishery.

LABID	SPECIES	TISSUE TYPE/COLLECTION TYPE	PRESERVATIVE/ESTIMATED TIME IN PRESERVATIVE
15501	Gray whale	Skin/Stranding	10% formalin/4.5 yrs
15635	Gray whale	Skin/Stranding	10% formalin/4.0 yrs
25507	Pacific white-sided	Skin/Fresh biopsy	10% formalin/3.0 yrs
18654	Bottlenose dolphin	Skin/Fresh biopsy	10% formalin/4.5 yrs
25503	Bottlenose dolphin	Skin/Fresh biopsy	10% formalin/3.0 yrs
26632	Common dolphin	Skin/Fresh biopsy	10% formalin/2.5 yrs
7220	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/32 yrs
7260	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/23 yrs
7306	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/13 yrs
7219	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/30 yrs
7235	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/14 yrs
7259	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/13 yrs
7264	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/12 yrs
7301	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/16 yrs
7307	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/13 yrs
7216	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/31 yrs
7302	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/16 yrs
7215	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/31 yrs
7303	Pantropical Spotted dolphin	Skin/ETP Fishery	10% formalin/3-10 mo, transferred to 70% isopropyl/16 yrs
159	Common dolphin	Skin/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/13 yrs
1865	Common dolphin	Skin/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/11 yrs
3863	Common dolphin	Skin/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/14 yrs
689	Common dolphin	Skin/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/13 yrs
1122	Common dolphin	Skin/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/12 yrs
694	Common dolphin	Skin/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/12 yrs
1131	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/13 yrs
1136	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/13 yrs
1122	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/12 yrs
1126	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/12 yrs
3863	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/14 yrs
230	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/13 yrs
4896	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/9 yrs
1865	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/11 yrs
694	Common dolphin	Tooth/CAGN Fishery	10% formalin/2-8 wks, transferred to 70% isopropyl/12 yrs

Table 2. Taq polymerases and PCR conditions tried on DNA extracted from formalin fixed tissues. Tissue type and amplification results are also summarized.

POLYMERASE	Invitrogen Platinum Taq	Stratagene PfuUltra Taq	Finnzymes Phusion Taq	Biolase Taq	Sigma Restorase Taq
Feature of polymerase	Increase yield, high specificity	High fidelity, less error	High fidelity, longer template	Robust yield	Repair enzyme
Reagents per reaction	Reagent:Volume	Reagent:Volume	Reagent:Volume	Reagent:Volume	Reagent:Volume
	10x Buffer:5ul 50M MgCl:3ul 10mM dNTP mix:3ul 10uM Primer1:1.5ul 10uM Primer2:1.5ul BSA:1ul Taq:0.5ul MQ H2O to 50ul	10x Pfu HF Buffer:5ul 50M MgC:0ul 10mM dNTP mix:3ul 10uM Primer1:1.5ul 10uM Primer2:1.5ul BSA:1ul Taq:1ul MQ H2O to 50ul	5x Phusion HF Buffer:10ul 50M MgCl:1.5ul 10mM dNTP mix:2ul 10uM Primer1:1.5ul 10uM Primer2:1.5ul BSA:0ul Taq:0.5ul MQ H2O to 50ul	10x Buffer:5ul 50M MgCl :2.5ul 10mM dNTP mix:3ul 10uM Primer1:1.5ul 10uM Primer2:1.5ul BSA:0ul Taq:0.5ul MQ H2O to 50ul	10x Restorase Buffer:5ul 50M MgCl:0ul 10mM dNTP mix:2ul 10uM Primer1:1.5ul 10uM Primer2:1.5ul BS :0ul Taq:1ul MQ H2O to 50ul
Tissue Type	skin	skin and teeth	skin	skin and teeth	teeth
Amplification?	yes	yes	yes	yes	no

PCR Conditions

Polymerase		1st DENATURE	DENATURE	ANNEALING	EXTEND	NO. CYCLES	FINAL EXTEND
Platinum	Temperature	95	94	48	72	35	72
	Time	15:00	:45	1:00	1:30		5:00
PfuUltra	Temperature	95	95	48	72	30	72
	Time	2:00	:30	:30	1:00		10:00
Phusion	Temperature	98	98	56	72	35	72
	Time	1:30	:30	:30	:30		5:00
Biolase	Temperature	94	94	48	72	50	72
	Time	2:30	:30	:45	1:30		10:00
Restorase	Temperature	94	94	48	68	30	68
	Time	:05	:05	:20	1:00		1:00

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Table 3. List of primer pairs used to amplify various regions of the 400 base mitochondrial control region sequences in formalin-fixed samples.

PRIMER PAIRS – NAME/SEQUENCE	REFERENCE	NUMBER BASES AMPLIFIED/ LOCATION ON 400 BASE SEQUENCE
TRO- 5'-CCTCCCTAAGACTCAAGGAAG- 3' D- 5'-CCTGAAGTAAGAACAGATG- 3'	Rosel et al. 1994 Developed SWFSC	400/entire
TRO- 5'-CCTCCCTAAGACTCAAGGAAG- 3' A3 - 5'-AATACGRGCTTTAACT- 3'	Developed SWFSC Developed SWFSC	240/beginning
TRO- 5'-CCTCCCTAAGACTCAAGGAAG- 3' SA1- 5'-ATGCATATTATGTAATATGTAA- 3'[1]	Developed SWFSC Developed SWFSC	260/beginning
D- 5'-CCTGAAGTAAGAACAGATG- 3' A3r- 5'-GATAAGTTA AAGCTCGTATT- 3'	Rosel et al. 1994 Developed SWFSC	270/end
D- 5'-CCTGAAGTAAGAACAGATG- 3' DL3c- 5'-GTGAAACCAGCAACCCGC- 3'	Rosel et al. 1994 Developed SWFSC	150/end
AD1- 5'-TTGCTGGTTTCACGCGG- 3' DL1- 5'-CATGCTATGTATAACTGTGCATTC- 3'	Developed SWFSC Developed SWFSC	210/middle
AD1- 5'-TTGCTGGTTTCACGCGG- 3' A3r- 5'-GATAAGTTA AAGCTCGTATT- 3'	Developed SWFSC Developed SWFSC	170/middle

[1] Species specific primer for spotted dolphin.

Table 4. Sequencing results for tissue type and extraction protocol.

LABID	COLLECTION TYPE/ PRESERVATIVE	EXTRACTION PROTOCOL	SEQUENCE OBTAINED
SKIN			
15501	stranding - formalin only	AR + QiaAMP Kit	**70 bases**
15635	stranding - formalin only	AR + QiaAMP Kit	**100 bases**
25507	biopsy - formalin only	AR + QiaAMP Kit	**110 bases**
18654	biopsy - formalin only	AR + QiaAMP Kit	amplified, poor sequence
25503	biopsy - formalin only	AR + QiaAMP Kit	failed
26632	biopsy - formalin only	AR + QiaAMP Kit	failed
7220	ETP, formalin and isopropyl	AR + QiaAMP Kit	failed
7220	ETP, formalin and isopropyl	Modified Höss and Pääbo	failed
7260	ETP, formalin and isopropyl	AR + QiaAMP Kit	**400 bases, with fragments**
7260	ETP, formalin and isopropyl	Modified Höss and Pääbo	failed **pieced together**
7306	ETP, formalin and isopropyl	AR + QiaAMP Kit	amplified, poor sequence
7306	ETP, formalin and isopropyl	Modified Höss and Pääbo	failed
7219	ETP, formalin and isopropyl	AR + QiaAMP Kit	failed
7235	ETP, formalin and isopropyl	AR + QiaAMP Kit	**210 bases**
7259	ETP, formalin and isopropyl	AR + QiaAMP Kit	failed
7264	ETP, formalin and isopropyl	AR + QiaAMP Kit	amplified, poor sequence
7301	ETP, formalin and isopropyl	AR + QiaAMP Kit	amplified, poor sequence
7307	ETP, formalin and isopropyl	AR + QiaAMP Kit	**180 bases**
7216	ETP, formalin and isopropyl	AR + QiaAMP Kit	failed
7302	ETP, formalin and isopropyl	AR + QiaAMP Kit	**210 bases**
7215	ETP, formalin and isopropyl	AR + QiaAMP Kit	failed
7303	ETP, formalin and isopropyl	AR + QiaAMP Kit	failed
159	CAGN, formalin and isopropyl	AR + QiaAMP Kit	**120 bases**
1865	CAGN, formalin and isopropyl	AR + QiaAMP Kit	failed
1865	CAGN, formalin and isopropyl	RecoverALL Kit	failed
3863	CAGN, formalin and isopropyl	RecoverALL Kit	failed
689	CAGN, formalin and isopropyl	AR + QiaAMP Kit	failed
1122	CAGN, formalin and isopropyl	AR + QiaAMP Kit	failed
1122	CAGN, formalin and isopropyl	RecoverALL Kit	failed
694	CAGN, formalin and isopropyl	AR + QiaAMP Kit	failed
694	CAGN, formalin and isopropyl	RecoverALL Kit	failed
TEETH			
1131	CAGN, formalin and isopropyl	Modified Höss and Pääbo	200 bases, poor quality
1136	CAGN, formalin and isopropyl	Modified Höss and Pääbo	200 bases, poor quality
1122	CAGN, formalin and isopropyl	Modified Höss and Pääbo	**400 bases**
1126	CAGN, formalin and isopropyl	Modified Höss and Pääbo	**180 bases**
3863	CAGN, formalin and isopropyl	Modified Höss and Pääbo	**200 bases**
230	CAGN, formalin and isopropyl	Modified Höss and Pääbo	**400 bases**
4896	CAGN, formalin and isopropyl	Modified Höss and Pääbo	**400 bases**
4896	CAGN, formalin and isopropyl	RecoverALL Kit	amplified, poor sequence
1865	CAGN, formalin and isopropyl	Modified Höss and Pääbo	**150 bases**
694	CAGN, formalin and isopropyl	Modified Höss and Pääbo	**200 bases**

Table 5. Base number and type of mutation found in the 400 base mitochondrial control region sequence from formalin fixed samples.

LAB#	42	117	148	150	154	191	246	279	280	281	286	290	291	300	301	359	390	TOTAL BASES OF SEQ
4896	C/T	G/A				G/A					C/T	C/T				G/A		400
230	C/T		G/A				G/A	C/T	C/T	C/T		C/T	C/T	G/A		G/A	C/T	400
1122					G/A		G/A			C/T			C/T		C/T			400
1865	C/T			G/A														only 1st 150 bases
3863							G/A	C/T	C/T	C/T			C/T	G/A		G/A	C/T	last 200 bases
694							G/A	C/T	C/T	C/T			C/T	G/A			C/T	last 200 bases

Figure 1. Example of an ambiguous base (blue highlighted area) represented in most sequences generated from formalin-fixed samples.

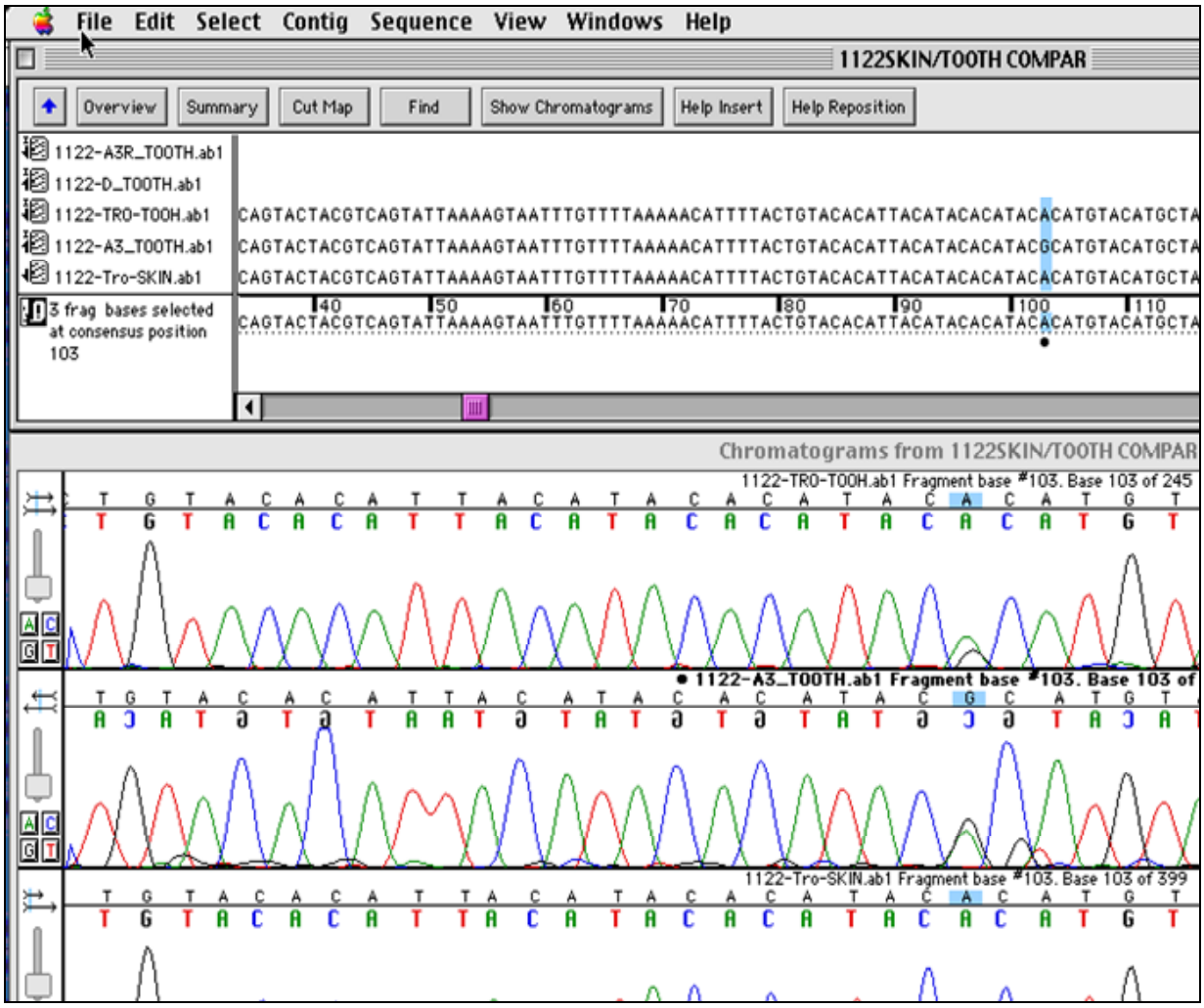


Figure 2. An insertion/deletion at base 120 (blue highlighted area) in a formalin tooth sequence. Comparison is made to the true base call in the sequence generated from DNA from the non-formalin preserved skin sample. Sequences from non-formalin preserved skin are top three rows and sequences from the formalin preserved tooth are bottom two rows.

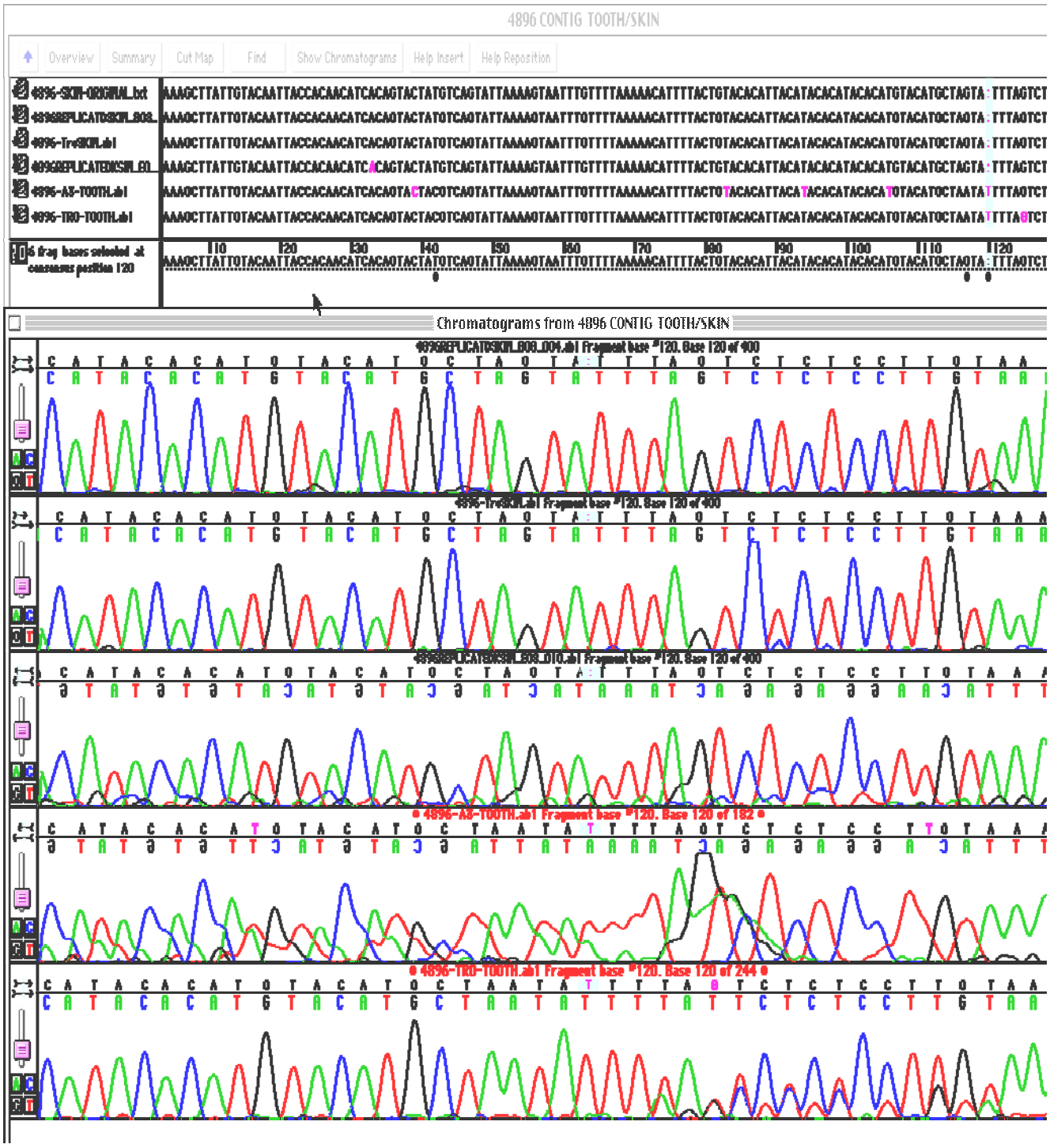
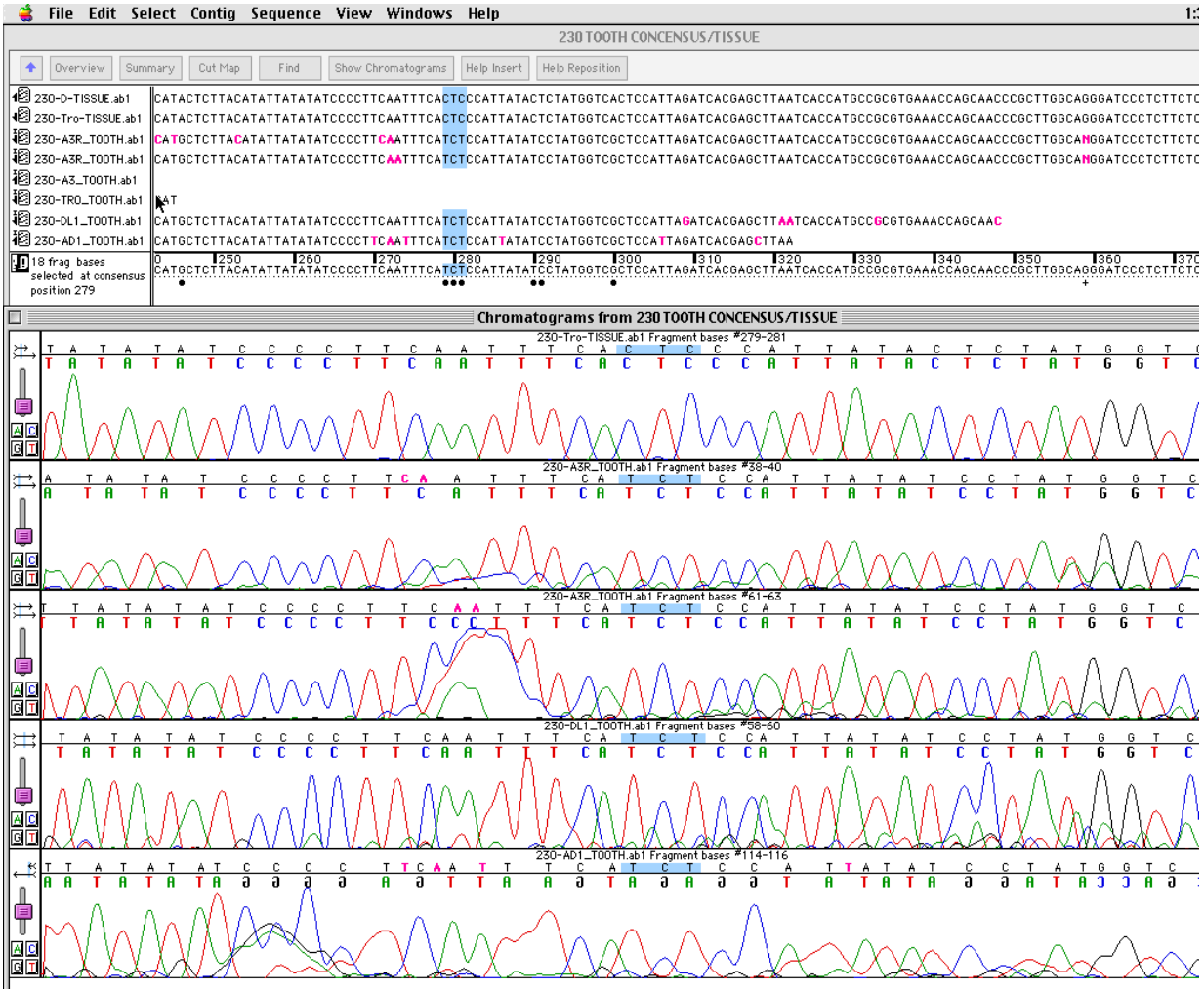


Figure 3. Chromatogram of sequence from tooth and skin samples from the same animal showing several C/T and A/G mutations (blue highlighted area). The top two sequences are generated from the non-formalin preserved skin and the bottom few are sequences generated from the formalin preserved tooth.



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